

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

THIS PAGE BLANK (USPTO)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 9/80, 15/00, 1/20, C12P 21/06, C07H 21/04, C07K 16/00	A1	(11) International Publication Number: WO 97/48794 (43) International Publication Date: 24 December 1997 (24.12.97)
(21) International Application Number: PCT/US97/09319 (22) International Filing Date: 17 June 1997 (17.06.97) (30) Priority Data: 08/664,646 17 June 1996 (17.06.96) US (71) Applicant: RECOMBINANT BIOCATALYSIS, INC. [US/US]; 505 East Coast Boulevard South, La Jolla, CA 92037 (US). (72) Inventors: MURPHY, Dennis; 10 Fairway Road, Paoli, PA 19301 (US). REID, John, C.; Apartment J-2, 922 Mont- gomery Avenue, Bryn Mawr, PA 19010 (US). ROBERT- SON, Dan; 33 Evergreen Lane, Haddonfield, NJ 08033 (US). (74) Agent: HAILE, Lisa, A.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: AMIDASE (57) Abstract A purified thermostable enzyme is derived from the archaeal bacterium <i>Thermococcus GUSL5</i> . The enzyme has a molecular weight of about 68.5 kilodaltons and has cellulase activity. The enzyme can be produced from native or recombinant host cells and can be used for the removal of arginine, phenylalanine, or methionine amino acids from the N-terminal end of peptides in peptide or peptidomimetic synthesis. The enzyme is selective for the L, or "natural" enantiomer of the amino acid derivatives and is therefore useful for the production of optically active compounds. These reactions can be performed in the presence of the chemically more reactive ester functionality, a step which is very difficult to achieve with nonenzymatic methods.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

AMIDASE

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production and isolation of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention has been identified as an amidase and in particular an enzyme having activity in the removal of arginine, phenylalanine or methionine from the N-terminal end of peptides in peptide or peptidomimetic synthesis.

Thermophilic bacteria have received considerable attention as sources of highly active and thermostable enzymes (Bronneomeier, K. and Staudenbauer, W.L., D.R. Woods (Ed.), The Clostridia and Biotechnology, Butterworth Publishers, Stoneham, MA (1993). Recently, the most extremely thermophilic organotrophic eubacteria presently known have been isolated and characterized. These bacteria, which belong to the genus *Thermotoga*, are fermentative microorganisms metabolizing a variety of carbohydrates (Huber, R. and Stetter, K.O., in Ballows, et al., (Ed.), The Prokaryotes, 2nd Ed., Springer-Verlag, New York, pgs. 3809-3819 (1992)).

Because to date most organisms identified from the archaeal domain are thermophiles or hyperthermophiles, archaeal bacteria are also considered a fertile source of thermophilic enzymes.

- 2 -

SUMMARY OF THE INVENTION

In accordance with one aspect of the present invention, there is provided a novel enzyme, as well as active fragments, analogs and derivatives thereof.

5 In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding an enzyme of the present invention including mRNAs, DNAs, cDNAs, genomic DNAs as well as active analogs and fragments of such enzymes.

10 In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence
15 encoding an enzyme of the present invention, under conditions promoting expression of said enzyme and subsequent recovery of said enzyme.

In accordance with yet a further aspect of the present invention, there is provided a process for
20 utilizing such enzyme, or polynucleotide encoding such enzyme. The enzyme is useful for the removal of arginine, phenylalanine, or methionine amino acids from the N-terminal end of peptides in peptide or peptidomimetic synthesis. The enzyme is selective for
25 the L, or "natural" enantiomer of the amino acid derivatives and is therefore useful for the production of optically active compounds. These reactions can be performed in the presence of the chemically more reactive ester functionality, a step which is very difficult to

- 3 -

achieve with nonenzymatic methods. The enzyme is also able to tolerate high temperatures (at least 70°C), and high concentrations of organic solvents (>40% DMSO), both of which cause a disruption of secondary structure in
5 peptides; this enables cleavage of otherwise resistant bonds.

In accordance with yet a further aspect of the present invention, there is also provided nucleic acid probes comprising nucleic acid molecules of sufficient
10 length to specifically hybridize to a nucleic acid sequence of the present invention.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes, or polynucleotides encoding such
15 enzymes, for *in vitro* purposes related to scientific research, for example, to generate probes for identifying similar sequences which might encode similar enzymes from other organisms.

These and other aspects of the present invention
20 should be apparent to those skilled in the art from the teachings herein.

- 4 -

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

5 Figure 1 is an illustration of the full-length DNA and corresponding deduced amino acid sequence of the enzyme of the present invention. Sequencing was performed using a 378 automated DNA sequencer (Applied Biosystems, Inc.).

10 Figure 2 shows the fluorescence versus concentration of DMSO. The filled and open boxes represent individual assays from Example 3.

 Figure 3 shows the relative initial linear rates (increase in fluorescence per min. i.e. "activity")
15 versus concentration of DMF for the more reactive CBZ-L-arg-AMC, from Example 3.

- 5 -

DETAILED DESCRIPTION OF THE INVENTION

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and
5 trailer) as well as intervening sequences (introns) between individual coding segments (exons).

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then
10 translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences are ultimately processed to produce the desired protein.

15 "Recombinant" enzymes refer to enzymes produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired enzyme. "Synthetic" enzymes are those prepared by chemical synthesis.

20 The present invention provides substantially pure amidase enzymes. The term "substantially pure" is used herein to describe a molecule, such as a polypeptide (e.g., an amidase polypeptide, or a fragment thereof) that is substantially free of other proteins, lipids,
25 carbohydrates, nucleic acids, and other biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a polypeptide, can be at least 60%, by dry weight, the molecule of interest. The purity of the polypeptides can

- 6 -

be determined using standard methods including, e.g., polyacrylamide gel electrophoresis (e.g., SDS-PAGE), column chromatography (e.g., high performance liquid chromatography (HPLC)), and amino-terminal amino acid
5 sequence analysis.

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular enzyme, is a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory
10 sequences. A "promotor sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The promoter is part of the DNA sequence. This sequence region has a start codon at its
15 3' terminus. The promoter sequence does include the minimum number of bases where elements necessary to initiate transcription at levels detectable above background. However, after the RNA polymerase binds the sequence and transcription is initiated at the start
20 codon (3' terminus with a promoter), transcription proceeds downstream in the 3' direction. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1) as well as protein binding domains
25 (consensus sequences) responsible for the binding of RNA polymerase.

The present invention provides a purified thermostable enzyme that catalyzes the removal of arginine, phenylalanine, or methionine amino acids from
30 the N-terminal end of peptides in peptide or peptidomimetic synthesis. The purified enzyme is an

- 7 -

amidase derived from an organism referred to herein as
"Thermococcus GU5L5" which is a thermophilic archaeal
organism which has a very high temperature optimum. The
organism is strictly anaerobic and grows between 55 and
5 90°C (optimally at 85°C). GU5L5 was discovered in a
shallow marine hydrothermal area in Vulcano, Italy. The
organism has coccoid cells occurring in singlets or
pairs. GU5L5 grows optimally at 85°C and pH 6.0 in a
marine medium with peptone as a substrate and nitrogen in
10 gas phase.

The polynucleotide of this invention was
originally recovered from a genomic gene library derived
from *Thermococcus* GU5L5 as described below. It contains
an open reading frame encoding a protein of 622 amino
15 acid residues.

In a preferred embodiment, the amidase enzyme of
the present invention has a molecular weight of about
68.5 kilodaltons as inferred from the nucleotide sequence
of the gene.

20 In accordance with an aspect of the present
invention, there are provided isolated nucleic acid
molecules (polynucleotides) which encode for the mature
enzyme having the deduced amino acid sequence of Figure 1
(SEQ ID NO:2).

25 This invention, in addition to the isolated
nucleic acid molecule encoding an amidase enzyme
disclosed in Figure 1 (SEQ ID NO:1), also provides
substantially similar sequences. Isolated nucleic acid
sequences are substantially similar if: (i) they are

- 8 -

capable of hybridizing under stringent conditions,
hereinafter described, to SEQ ID NO:1; or (ii) they
encode DNA sequences which are degenerate to SEQ ID NO:1.
Degenerate DNA sequences encode the amino acid sequence
5 of SEQ ID NO:2, but have variations in the nucleotide
coding sequences. As used herein, "substantially
similar" refers to the sequences having similar identity
to the sequences of the instant invention. The
nucleotide sequences that are substantially similar can
10 be identified by hybridization or by sequence comparison.
Enzyme sequences that are substantially similar can be
identified by one or more of the following: proteolytic
digestion, gel electrophoresis and/or microsequencing.

One means for isolating a nucleic acid molecule
15 encoding an amidase enzyme is to probe a gene library
with a natural or artificially designed probe using art
recognized procedures (see, for example: Current
Protocols in Molecular Biology, Ausubel F.M. et al.
(EDS.) Green Publishing Company Assoc. and John Wiley
20 Interscience, New York, 1989, 1992). It is appreciated
to one skilled in the art that SEQ ID NO:1, or fragments
thereof (comprising at least 15 contiguous nucleotides),
is a particularly useful probe. Other particular useful
probes for this purpose are hybridizable fragments to the
25 sequences of SEQ ID NO:1 (i.e., comprising at least 15
contiguous nucleotides).

With respect to nucleic acid sequences which
hybridize to specific nucleic acid sequences disclosed
herein, hybridization may be carried out under conditions
30 of reduced stringency, medium stringency or even
stringent conditions. As an example of oligonucleotide

- 9 -

hybridization, a polymer membrane containing immobilized denatured nucleic acid is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH₂PO₄, pH 7.0, 5.0 mM Na₂EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/mL polyriboadenylic acid. Approximately 2×10^7 cpm (specific activity $4-9 \times 10^8$ cpm/ug) of ³²P end-labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na₂EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at T_m-10°C for the oligo-nucleotide probe. The membrane is then exposed to auto-radiographic film for detection of hybridization signals.

Stringent conditions means hybridization will occur only if there is at least 90% identity, preferably at least 95% identity and most preferably at least 97% identity between the sequences. See J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory) which is hereby incorporated by reference in its entirety.

"Identity" as the term is used herein, refers to a polynucleotide sequence which comprises a percentage of the same bases as a reference polynucleotide (SEQ ID NO:1). For example, a polynucleotide which is at least 90% identical to a reference polynucleotide, has polynucleotide bases which are identical in 90% of the bases which make up the reference polynucleotide and may have different bases in 10% of the bases which comprise that polynucleotide sequence.

- 10 -

The present invention also relates to polynucleotides which differ from the reference polynucleotide such that the changes are silent changes, for example the changes do not alter the amino acid
5 sequence encoded by the polynucleotide. The present invention also relates to nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations in the enzyme encoded by the reference polynucleotide (SEQ ID NO:1). In a preferred
10 aspect of the invention these enzymes retain the same biological action as the enzyme encoded by the reference polynucleotide.

It is also appreciated that such probes can be and are preferably labeled with an analytically detectable
15 reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactivity, fluorescent dyes or enzymes capable of catalyzing the formation of a detectable product. The probes are thus useful to isolate complementary copies of
20 DNA from other animal sources or to screen such sources for related sequences.

The coding sequence for the amidase enzyme of the present invention was identified by preparing a *Thermococcus* GU5L5 genomic DNA library and screening the
25 library for the clones having amidase activity. Such methods for constructing a genomic gene library are well-known in the art. One means, for example, comprises shearing DNA isolated from GU5L5 by physical disruption. A small amount of the sheared DNA is checked on an
30 agarose gel to verify that the majority of the DNA is in the desired size range (approximately 3-6 kb). The DNA

- 11 -

is then blunt ended using Mung Bean Nuclease, incubated at 37°C and phenol/chloroform extracted. The DNA is then methylated using Eco RI Methylase. Eco RI linkers are then ligated to the blunt ends through the use of T4 DNA
5 ligase and incubation at 4°C. The ligation reaction is then terminated and the DNA is cut-back with Eco RI restriction enzyme. The DNA is then size fractionated on a sucrose gradient following procedures known in the art, for example, Maniatis, T., et al., Molecular Cloning,
10 Cold Spring Harbor Press, New York, 1982, which is hereby incorporated by reference in its entirety.

A plate assay is then performed to get an approximate concentration of the DNA. Ligation reactions are then performed and 1 µl of the ligation reaction is
15 packaged to construct a library. Packaging, for example, may occur through the use of purified λgt11 phage arms cut with EcoRI and DNA cut with EcoRI after attaching EcoRI linkers. The DNA and λgt11 arms are ligated with DNA ligase. The ligated DNA is then packaged into
20 infectious phage particles. The packaged phages are used to infect *E. coli* cultures and the infected cells are spread on agar plates to yield plates carrying thousands of individual phage plaques. The library is then amplified.

25 Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA or a genomic library to isolate the full length DNA and to isolate other DNAs which have a high sequence similarity to the gene or similar biological activity.
30 Probes of this type have at least 10, preferably at least 15, and even more preferably at least 30 bases and may

- 12 -

contain, for example, at least 50 or more bases. The probe may also be used to identify a DNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including
5 regulatory and promotor regions, exons, and introns.

The isolated nucleic acid sequences and other enzymes may then be measured for retention of biological activity characteristic to the enzyme of the present invention, for example, in an assay for detecting
10 enzymatic amidase activity. Such enzymes include truncated forms of amidase, and variants such as deletion and insertion variants.

The polynucleotide of the present invention may be in the form of DNA which DNA includes cDNA, genomic DNA,
15 and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature enzyme may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:1)
20 and/or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature enzyme as the DNA of Figure 1 (SEQ ID NO:1).

The polynucleotide which encodes for the mature
25 enzyme of Figure 1 (SEQ ID NO:2) may include, but is not limited to: only the coding sequence for the mature enzyme; the coding sequence for the mature enzyme and additional coding sequence such as a leader sequence or a proprotein sequence; the coding sequence for the mature
30 enzyme (and optionally additional coding sequence) and

- 13 -

non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature enzyme.

Thus, the term "polynucleotide encoding an enzyme
5 (protein)" encompasses a polynucleotide which includes only coding sequence for the enzyme as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants
10 of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the enzyme having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2). The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide
15 or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature enzyme as shown in Figure 1 (SEQ ID NO:2) as well as variants of such
20 polynucleotides which variants encode for a fragment, derivative or analog of the enzyme of Figure 1 (SEQ ID NO:2). Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

25 As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID NO:1). As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which

- 14 -

may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded enzyme.

The present invention also includes
5 polynucleotides, wherein the coding sequence for the mature enzyme may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of an enzyme from a host cell, for example, a leader sequence which functions to control transport of
10 an enzyme from the cell. The enzyme having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the enzyme. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5'
15 amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the
20 present invention may encode for a mature enzyme, or for an enzyme having a prosequence or for an enzyme having both a prosequence and a presequence (leader sequence).

The present invention further relates to polynucleotides which hybridize to the hereinabove-
25 described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described
30 polynucleotides. As herein used, the term "stringent

- 15 -

conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode enzymes which either retain substantially the same biological function or activity as the mature enzyme encoded by the DNA of Figure 1 (SEQ ID NO:1).

Alternatively, the polynucleotide may have at least 15 bases, preferably at least 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% identity and more preferably at least a 95% identity to a polynucleotide which encodes the enzyme of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to enzymes encoded by such polynucleotides.

The present invention further relates to a enzyme which has the deduced amino acid sequence of Figure 1 (SEQ ID NO:2), as well as fragments, analogs and derivatives of such enzyme.

- 16 -

The terms "fragment," "derivative" and "analog" when referring to the enzyme of Figure 1 (SEQ ID NO:2) means a enzyme which retains essentially the same biological function or activity as such enzyme. Thus, an
5 analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature enzyme.

The enzyme of the present invention may be a recombinant enzyme, a natural enzyme or a synthetic
10 enzyme, preferably a recombinant enzyme.

The fragment, derivative or analog of the enzyme of Figure 1 (SEQ ID NO:2) may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably
15 a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature enzyme is fused with
20 another compound, such as a compound to increase the half-life of the enzyme (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature enzyme, such as a leader or secretory sequence or a sequence which is employed for
25 purification of the mature enzyme or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

- 17 -

The enzymes and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The enzymes of the present invention include the enzyme of SEQ ID NO:2 (in particular the mature enzyme) as well as enzymes which have at least 70% similarity (preferably at least 70% identity) to the enzyme of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the enzyme of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the enzyme of SEQ ID NO:2 and also include portions of such enzymes with such portion of the enzyme generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two enzymes is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one enzyme to the sequence of a second enzyme. Similarity

- 18 -

may be determined by procedures which are well-known in the art, for example, a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information).

5 A variant, i.e. a "fragment", "analog" or "derivative" enzyme, and reference enzyme may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

10 Among preferred variants are those that vary from a reference by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative
15 substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the
20 basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Most highly preferred are variants which retain the same biological function and activity as the reference polypeptide from which it varies.

25 Fragments or portions of the enzymes of the present invention may be employed for producing the corresponding full-length enzyme by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length enzymes. Fragments or

- 19 -

portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors
5 which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of enzymes of the invention by recombinant techniques.

Host cells are genetically engineered (transduced
10 or transformed or transfected) with the vectors containing the polynucleotides of this invention. Such vectors may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc.
15 The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those
20 previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing enzymes by recombinant
25 techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing an enzyme. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA;
30 baculovirus; yeast plasmids; vectors derived from

- 20 -

combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

5 The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the
10 scope of those skilled in the art.

 The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be
15 mentioned: LTR or SV40 promoter, the *E. coli*. *lac* or *trp*, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation
20 initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

 In addition, the expression vectors preferably contain one or more selectable marker genes to provide a
25 phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

 The vector containing the appropriate DNA sequence

- 21 -

as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

5 As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Bacillus subtilis*; fungal cells, such as yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS or Bowes melanoma;
10 adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of
15 the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further
20 comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example;
25 Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBluescript II (Stratagene); pTRC99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other
30 plasmid or vector may be used as long as they are replicable and viable in the host.

- 22 -

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named
5 bacterial promoters include *lacI*, *lacZ*, T3, T7, *gpt*,
lambda P_R, *P_L* and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well
10 within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic
15 cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Digner, M.,
20 Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the enzymes of the invention can be synthetically produced by
25 conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs
30 derived from the DNA constructs of the present invention.

- 23 -

Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the enzymes of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about 10 from 10 to 300 bp that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and 15 adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* 20 TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α -factor, acid phosphatase, or heat shock 25 proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated enzyme. Optionally, the 30 heterologous sequence can encode a fusion enzyme including an N-terminal identification peptide imparting

- 24 -

desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence
5 encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure
10 maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and
15 *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication
20 derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA).
25 These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell
30 density, the selected promoter is induced by appropriate

- 25 -

means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the
5 resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption,
10 or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of
15 monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable
20 promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites
25 may be used to provide the required nontranscribed genetic elements.

The enzyme can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion

- 26 -

or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can
5 be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The enzymes of the present invention may be a
10 naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host
15 employed in a recombinant production procedure, the enzymes of the present invention may be glycosylated or may be non-glycosylated. Enzymes of the invention may or may not also include an initial methionine amino acid residue.

20 The enzymes, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes
25 chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the enzymes
30 corresponding to a sequence of the present invention can

- 27 -

be obtained by direct injection of the enzymes into an animal or by administering the enzymes to an animal, preferably a nonhuman. The antibody so obtained will then bind the enzymes itself. In this manner, even a
5 sequence encoding only a fragment of the enzymes can be used to generate antibodies binding the whole native enzymes. Such antibodies can then be used to isolate the enzyme from cells expressing that enzyme.

For preparation of monoclonal antibodies, any
10 technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983,
15 Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single
20 chain antibodies (U.S. Patent 4 946,778) can be adapted to produce single chain antibodies to immunogenic enzyme products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic enzyme products of this invention.

25 Antibodies generated against the enzyme of the present invention may be used in screening for similar enzymes from other organisms and samples. Such screening techniques are known in the art, for example, one such screening assay is described in "Methods for Measuring
30 Cellulase Activities", *Methods in Enzymology*, Vol 160,

- 28 -

pp. 37-116, which is hereby incorporated by reference in its entirety. Antibodies may also be employed as a probe to screen gene libraries generated from this or other organisms to identify this or cross reactive activities.

- 5 The term "antibody," as used herein, refers to intact immunoglobulin molecules, as well as fragments of immunoglobulin molecules, such as Fab, Fab', (Fab')₂, Fv, and SCA fragments, that are capable of binding to an epitope of an amidase polypeptide. These antibody
- 10 fragments, which retain some ability to selectively bind to the antigen (e.g., an amidase antigen) of the antibody from which they are derived, can be made using well known methods in the art (see, e.g., Harlow and Lane, *supra*), and are described further, as follows.
- 15 (1) A Fab fragment consists of a monovalent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain.
- 20 (2) A Fab' fragment of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per
- 25 antibody molecule treated in this manner.
- (3) A (Fab')₂ fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme

- 29 -

pepsin, without subsequent reduction. A (Fab')₂ fragment is a dimer of two Fab' fragments, held together by two disulfide bonds.

(4) An Fv fragment is defined as a genetically engineered
5 fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains.

(5) A single chain antibody ("SCA") is a genetically
engineered single chain molecule containing the variable
10 region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

As used in this invention, the term "epitope" refers to an antigenic determinant on an antigen, such as
15 an amidase polypeptide, to which the paratope of an antibody, such as an amidase-specific antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-
20 dimensional structural characteristics, as well as specific charge characteristics.

The present invention is further described with reference to the following examples; however, it is to be understood that the present invention is not limited to
25 such examples. All parts or amounts, unless otherwise specified, are by weight.

- 30 -

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

- 31 -

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single
5 stranded polydeoxynucleotide or two complementary
polydeoxynucleotide strands which may be chemically
synthesized. Such synthetic oligonucleotides may or may
not have a 5' phosphate. Those that do not will not
ligate to another oligonucleotide without adding a
10 phosphate with an ATP in the presence of a kinase. A
synthetic oligonucleotide will ligate to a fragment that
has not been dephosphorylated.

"Ligation" refers to the process of forming
phosphodiester bonds between two double stranded nucleic
15 acid fragments (Maniatis et al., *Id.*, p. 146). Unless
otherwise provided, ligation may be accomplished using
known buffers and conditions with 10 units of T4 DNA
ligase ("ligase") per 0.5 µg of approximately equimolar
amounts of the DNA fragments to be ligated.

20 Unless otherwise stated, transformation was
performed as described in the method of Sambrook, Fritsch
and Maniatus, 1989.

Example 1

Bacterial Expression and Purification of Amidase

25 A *Thermococcus* GU5L5 genomic library was screened
for amidase activity as described in Example 2 and a
positive clone was identified and isolated. DNA of this
clone was used as a template in a 100 µl PCR reaction
using the following primer sequences:

- 32 -

5' primer: CCGAGAATTC ATTAAAGAGG AGAAATTAAC TATGACCGGC
ATCGAATGGA 3' (SEQ ID NO:3). 3' primer: 5' AATAAGGATC
CACACTGGCA CAGTGTCAAG ACA 3' (SEQ ID NO:4).

The protein was expressed in *E. coli*. The gene
5 was amplified using PCR with the primers indicated above.

Subsequent to amplification, the PCR product was
cloned into the *EcoRI* and *BamHI* sites of pQET1 and
transformed by electroporation into *E. coli* M15(pREP4).
The resulting transformants were grown up in 3ml
10 cultures, and a portion of this culture was induced. A
portion of the uninduced and induced cultures were
assayed using 2-L-Phe-AMC (see below).

The primer sequences set out above may also be
employed to isolate the target gene from the deposited
15 material by hybridization techniques described above.

Example 2

Discovery of an amidase from *Thermococcus* GU5L5

Production of the expression gene bank.

Colonies containing pBluescript plasmids with
20 random inserts from the organism *Thermococcus* GU5L5 was
obtained according to the method of Hay and Short. (Hay,
B. and Short, J., *Strategies*. 1992, 5, 16.) The
resulting colonies were picked with sterile toothpicks
and used to singly inoculate each of the wells of 96-well
25 microtiter plates. The wells contained 250 μ L of LB
media with 100 μ g/mL ampicillin, 80 μ g/mL methicillin,
and 10% v/v glycerol (LB Amp/Meth, glycerol). The cells
were grown overnight at 37°C without shaking. This

- 33 -

constituted generation of the "SourceGeneBank"; each well of the Source GeneBank thus contained a stock culture of *E. coli* cells, each of which contained a pBluescript plasmid with a unique DNA insert.

5 Screening for amidase activity.

- The plates of the Source GeneBank were used to multiply inoculate a single plate (the "Condensed Plate") containing in each well 200 μ L of LB Amp/Meth, glycerol. This step was performed using the High Density
- 10 Replicating Tool (HDRT) of the Beckman Biomek with a 1% bleach, water, isopropanol, air-dry sterilization cycle in between each inoculation. Each well of the Condensed Plate thus contained 10 to 12 different pBluescript clones from each of the source library plates. The
- 15 Condensed Plate was grown for 16h at 37°C and then used to inoculate two white 96-well Polyfiltronics microtiter daughter plates containing in each well 250 μ L of LB Amp/Meth (without glycerol). The original condensed plate was put in storage -80°C. The two condensed
- 20 daughter plates were incubated at 37°C for 18 h.

- The '600 μ M substrate stock solution' was prepared as follows: 25 mg of N-morphourea-L-phenylalanyl-7-amido-4-trifluoromethylcoumarin (Mu-Phe-AFC, Enzyme Systems Products, Dublin, CA) was dissolved in the
- 25 appropriate volume of DMSO to yield a 25.2 mM solution. Two hundred fifty microliters of DMSO solution was added to ca. 9 mL of 50 mM, pH 7.5 Hepes buffer containing 0.6 mg/mL of dodecyl maltoside. The volume was taken to 10.5 mL with the above Hepes buffer to yield a cloudy
- 30 solution.

- 34 -

Mu-Phe-AFC

Fifty μ L of the '600 μ M stock solution' was added to each of the wells of a white condensed plate using the Biomek to yield a final concentration of substrate of 5 ~100 μ M. The fluorescence values were recorded (excitation = 400 nm, emission = 505 nm) on a plate reading fluorometer immediately after addition of the substrate. The plate was incubated at 70°C for 60 min. and the fluorescence values were recorded again. The 10 initial and final fluorescence values were subtracted to determine if an active clone was present by an increase in fluorescence over the majority of the other wells.

Isolation of the active clone.

In order to isolate the individual clone which 15 carried the activity, the Source GeneBank plates were thawed and the individual wells used to singly inoculate a new plate containing LB Amp/Meth. As above the plate was incubated at 37°C to grow the cells, and 50 μ L of 600 μ M substrate stock solution added using the Biomek. Once 20 the active well from the source plate was identified, the cells from the source plate were used to inoculate 3mL cultures of LB/AMP/Meth, which were grown overnight. The plasmid DNA was isolated from the cultures and utilized for sequencing and construction of expression subclones.

25

Example 3**Thermococcus GU5L5 Amidase characterization****Substrate specificity.**

Using the following substrates (see below for definitions of the abbreviations): CBZ-L-ala-AMC, CBZ-L-

- 35 -

arg-AMC, CBZ-L-met-AMC, CBZ-L-phe-AMC, and 7-methyl-umbelliferyl heptanoate at 100 μ M for 1 hour at 70°C in the assays as described in the clone discovery section, the relative activity of the amidase was 3:3:1:<0.1: <0.1
5 for the compounds CBZ-L-arg-AMC : CBZ-L-phe-AMC : CBZ-L-met-AMC : CBZ-L-ala-AMC : 7-methylumbelliferyl heptanoate. The excitation and emission wavelengths for the 7-amido-4-methylcoumarins were 380 and 460 nm respectively, and 326 and 450 for the
10 methylumbelliferone.

The abbreviations stand for the following compounds:

CBZ-L-ala-AMC = N α -carbonylbenzyloxy-L-alanine-7-amido-4-methylcoumarin
15 CBZ-L-arg-AMC = N α -carbonylbenzyloxy-L-arginine-7-amido-4-methylcoumarin
CBZ-D-arg-AMC = N α -carbonylbenzyloxy-D-arginine-7-amido-4-methylcoumarin
CBZ-L-met-AMC = N α -carbonylbenzyloxy-L-methionine-
20 7-amido-4-methylcoumarin
CBZ-L-phe-AMC = N α -carbonylbenzyloxy-L-phenylalanine-7-amido-4-methylcoumarin

Organic solvent sensitivity.

The activity of the amidase in increasing
25 concentrations of dimethyl sulfoxide (DMSO) was tested as follows: to each well of a microtiter plate was added 10 μ L of 3 mM CBZ-L-phe-AMC in DMSO, 25 μ L of cell lysate containing the amidase activity, and 250 μ L of a variable mixture of DMSO:pH 7.5, 50 mM Hepes buffer. The
30 reactions were heated for 1 hour at 70°C and the

- 36 -

fluorescence measured. Figure 2 shows the fluorescence versus concentration of DMSO. The filled and open boxes represent individual assays.

The activity and enantioselectivity of the amidase
5 in increasing concentrations of dimethyl formamide (DMF)
was tested as follows: to each well of a microtiter
plate was added 30 μ L of 1 mM CBZ-L-arg-AMC or CBZ-D-arg-
AMC in DMF, 30 μ L of cell lysate containing the amidase
activity, and 240 μ L of a variable mixture of DMF:pH 7.5,
10 50 mM Hepes buffer. The reactions were incubated at RT
for 1 hour and the fluorescence measured at 1 minute
intervals. Figure 3 shows the relative initial linear
rates (increase in fluorescence per min, i.e.,
'activity') versus concentration of DMF for the more
15 reactive CBZ-L-arg-AMC.

The initial linear rate ('activity') of the L and
the D CBZ-arg-AMC substrates are shown in Tables 1 and 2
below:

Table 1

20 Activity of the CBZ-L-
arg-AMC:

DMF	Initial Rate, Fl.U./min
0.4%	654
10%	2548
20%	1451
30%	541
40%	345

Table 2

Activity of the CBZ-D-
arg-AMC:

DMF	Initial Rate, Fl.U./min
0.4%	0.3
10%	10.1
20%	4.6
30%	1.8
40%	0.9

- 37 -

50%	303
60%	190
75%	81
90%	11

50%	1.2
60%	1.4
75%	0.1
90%	0.1

5 The above data indicate that the enzyme shows excellent selectivity for the L, or 'natural' enantiomer of the derivatized amino acid substrate.

 Numerous modifications and variations of the present invention are possible in light of the above
10 teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

- 38 -

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
 - (i) APPLICANT: Recombinant Biocatalysis, Inc.
 - (ii) TITLE OF INVENTION: Amidases
 - (iii) NUMBER OF SEQUENCES: 4
 - (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: FISH & RICHARDSON
 - (B) STREET: 4225 EXECUTIVE SQUARE, STE. 1400
 - (C) CITY: LA JOLLA
 - (D) STATE: CA
 - (E) COUNTRY: USA
 - (F) ZIP: 92037
 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: 3.5 INCH DISKETTE
 - (B) COMPUTER: IBM PS/2
 - (C) OPERATING SYSTEM: MS-DOS
 - (D) SOFTWARE: WORD PERFECT 6.0
 - (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: Unassigned
 - (B) FILING DATE: Herewith
 - (C) CLASSIFICATION:
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/664,646
 - (B) FILING DATE: 17 June 1996
 - (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: LISA A. HAILE, Ph.D.
 - (B) REGISTRATION NUMBER: 38,347
 - (C) REFERENCE/DOCKET NUMBER: 09010/005WO1
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 619-678-5070
 - (B) TELEFAX: 619-678-5099

- 39 -

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS
 (A) LENGTH: 1869 NUCLEOTIDES
 (B) TYPE: NUCLEIC ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG ACC GGC ATC GAA TGG AAC CAC GAG ACC TTT TCT AAG TTC GCC TAC	48
Met Thr Gly Ile Glu Trp Asn His Glu Thr Phe Ser Lys Phe Ala Tyr	
5 10 15	
CTG GGC GAC CCG AGG ATA CGG GGA AAC TTA ATC GCG TAC ACC CTG ACG	96
Leu Gly Asp Pro Arg Ile Arg Gly Asn Leu Ile Ala Tyr Thr Leu Thr	
20 25 30	
AAG GCC AAC ATG AAG GAC AAC AAG TAC GAG AGC ACG GTT GTT GTT GAA	144
Lys Ala Asn Met Lys Asp Asn Lys Tyr Glu Ser Thr Val Val Val Glu	
35 40 45	
GAC CTT GAA ACG GGC TCA AGG CGC TTC ATC GAG AAC GCC TCA ATG CCG	192
Asp Leu Glu Thr Gly Ser Arg Arg Phe Ile Glu Asn Ala Ser Met Pro	
50 55 60	
AGG ATT TCG CCA GAC GGC AGA AAG CTC GCC TTC ACC TGC TTT AAC GAG	240
Arg Ile Ser Pro Asp Gly Arg Lys Leu Ala Phe Thr Cys Phe Asn Glu	
65 70 75 80	
GAG AAG AAG GAG ACC GAG ATA TGG GTG GCC GAT ATC CAG ACC CTG AGC	288
Glu Lys Lys Glu Thr Glu Ile Trp Val Ala Asp Ile Gln Thr Leu Ser	
85 90 95	
GCC AAG AAA GTC CTC TCA ACT AAA AAC GTC CGC TCG ATG CAG TGG AAC	336
Ala Lys Lys Val Leu Ser Thr Lys Asn Val Arg Ser Met Gln Trp Asn	
100 105 110	
GAC GAT TCA AGG AGA CTC TTA GTT GTC GGC TTC AAG AGG AGG GAC GAT	384
Asp Asp Ser Arg Arg Leu Leu Val Val Gly Phe Lys Arg Arg Asp Asp	
115 120 125	
GAG GAC TTC GTC TTT GAC GAC GAC GTC CCG GTC TGG TTC GAC AAT ATG	432
Glu Asp Phe Val Phe Asp Asp Val Pro Val Trp Phe Asp Asn Met	
130 135 140	
GGA TTC TTT GAT GGA GAG AAG ACG ACG TTC TGG GTT CTT GAC ACT GAG	480
Gly Phe Phe Asp Gly Glu Lys Thr Thr Phe Trp Val Leu Asp Thr Glu	
145 150 155 160	
GCC GAG GAG ATA ATC GAG CAG TTC GAG AAG CCG AGG TTT TCG AGT GGC	528
Ala Glu Glu Ile Ile Glu Gln Phe Glu Lys Pro Arg Phe Ser Ser Gly	
165 170 175	
CTC TGG CAC GGC GAT GCG ATA GTT GTG AAC GTC CCG CAC CGC GAG GGG	576
Leu Trp His Gly Asp Ala Ile Val Val Asn Val Pro His Arg Glu Gly	
180 185 190	

- 40 -

AGC AAG CCT GCC CTG TTC AAG TTC TAC GAC ATA GTC CTA TGG AAG GAC Ser Lys Pro Ala Leu Phe Lys Phe Tyr Asp Ile Val Leu Trp Lys Asp 195 200 205	624
GGG GAG GAA GAG AAG CTC TTC GAG AGG GTC TCC TTC GAG GCG GTT GAC Gly Glu Glu Glu Lys Leu Phe Glu Arg Val Ser Phe Glu Ala Val Asp 210 215 220	672
TCC GAC GGA AAG AGA ATA CTC CTG AGG GGC AAG AAA AAA AAG CGG TTC Ser Asp Gly Lys Arg Ile Leu Leu Arg Gly Lys Lys Lys Arg Phe 225 230 235 240	720
ATC AGC GAG CAC GAC TGG CTG TAC CTC TGG GAC GGC GAG CTT AAA CCG Ile Ser Glu His Asp Trp Leu Tyr Leu Trp Asp Gly Glu Leu Lys Pro 245 250 255	768
ATC TAC GAG GGC CCG CTC GAC GTC TGG GAA GCC AAG CTC ACG GAA GGA Ile Tyr Glu Gly Pro Leu Asp Val Trp Glu Ala Lys Leu Thr Glu Gly 260 265 270	816
AAG GTC TAC TTC CTC ACT CCA GAT GCG GGC AGG GTA AAC CTC TGG CTC Lys Val Tyr Phe Leu Thr Pro Asp Ala Gly Arg Val Asn Leu Trp Leu 275 280 285	864
TGG GAC GGG AAG GCC GAG CGT GTT GTT ACC GGC GAC CAC TGG ATT TAC Trp Asp Gly Lys Ala Glu Arg Val Val Thr Gly Asp His Trp Ile Tyr 290 295 300	912
GGG CTT GAC GTC AGC GAT GGC AAA GCA TTG CTC CTC ATC ATG ACC GCC Gly Leu Asp Val Ser Asp Gly Lys Ala Leu Leu Leu Ile Met Thr Ala 305 310 315 320	960
ACG AGG ATA GGC GAG CTC TAC CTC TAC GAC GGC GAG CTG AAA CAG GTC Thr Arg Ile Gly Glu Leu Tyr Leu Tyr Asp Gly Glu Leu Lys Gln Val 325 330 335	1008
ACC GAA TAC AAC GGG CCG ATA TTC AGG AAG CTC AAG ACC TTC GAG CCG Thr Glu Tyr Asn Gly Pro Ile Phe Arg Lys Leu Lys Thr Phe Glu Pro 340 345 350	1056
AGG CAC TTC CGC TTC AAG AGC AAA GAC CTC GAG ATA GAC GGC TGG TAC Arg His Phe Arg Phe Lys Ser Lys Asp Leu Glu Ile Asp Gly Trp Tyr 355 360 365	1104
CTC AGG CCG GAG GTT AAA GAG GAG AAG GCC CCG GTG ATA GTC TTC GTC Leu Arg Pro Glu Val Lys Glu Glu Lys Ala Pro Val Ile Val Phe Val 370 375 380	1152
CAC GGC GGG CCG AAG GGC ATG TAC GGA CAC CGC TTC GTC TAC GAG ATG His Gly Gly Pro Lys Gly Met Tyr Gly His Arg Phe Val Tyr Glu Met 385 390 395 400	1200
CAG CTG ATG GCG AGC AAG GGC TAC TAC TGC TGC TTC GTG AAC CCG CGC Gln Leu Met Ala Ser Lys Gly Tyr Tyr Val Val Phe Val Asn Pro Arg 405 410 415	1248
GGC AGC GAC GGC TAT AGC GAA GAC TTC GCG CTC CGC GTC CTG GAG AGG Gly Ser Asp Gly Tyr Ser Glu Asp Phe Ala Leu Arg Val Leu Glu Arg 420 425 430	1296

- 41 -

ACT GGC TTG GAG GAC TTT GAG GAC ATA ATG AAC GGC ATC GAG GAG TTC	1344
Thr Gly Leu Glu Asp Phe Glu Asp Ile Met Asn Gly Ile Glu Glu Phe	
435 440 445	
TTC AAG CTC GAA CCG CAG GCC GAC AGG GAG CGC GTT GGA ATA ACG GGC	1392
Phe Lys Leu Glu Pro Gln Ala Asp Arg Glu Arg Val Gly Ile Thr Gly	
450 455 460	
ATA AGC TAC GGC GGC TTC ATG ACC AAC TGG GCC TTG ACT CAG AGC GAC	1440
Ile Ser Tyr Gly Gly Phe Met Thr Asn Trp Ala Leu Thr Gln Ser Asp	
465 470 475 480	
CTC TTC AAG GCA GGA ATA AGC GAG AAC GGC ATA AGC TAC TGG CTC ACC	1488
Leu Phe Lys Ala Gly Ile Ser Glu Asn Gly Ile Ser Tyr Trp Leu Thr	
485 490 495	
AGC TAC GCC TTC TCG GAC ATA GGG CTC TGG TAC GAC GTC GAG GTC ATC	1536
Ser Tyr Ala Phe Ser Asp Ile Gly Leu Trp Tyr Asp Val Glu Val Ile	
500 505 510	
GGG CCA AAT CCG TTA GAG AAC GAG AAC TTC AGG AAG CTC AGC CCG CTG	1584
Gly Pro Asn Pro Leu Glu Asn Glu Asn Phe Arg Lys Leu Ser Pro Leu	
515 520 525	
TTC TAC GCT CAG AAC GTG AAG GCG CCG ATA CTC CTA ATC CAC TCG CTT	1632
Phe Tyr Ala Gln Asn Val Lys Ala Pro Ile Leu Ile His Ser Leu	
530 535 540	
GAG GAC TAC CGC TGT CCG CTC GAC CAG AGC CTT ATG TTC TAC AAC GTG	1680
Glu Asp Tyr Arg Cys Pro Leu Asp Gln Ser Leu Met Phe Tyr Asn Val	
545 550 555 560	
CTC AAG GAC ATG GGC AAG GAA GCC TAC ATA GCG ATA TTC AAG CGC GGC	1728
Leu Lys Asp Met Gly Lys Glu Ala Tyr Ile Ala Ile Phe Lys Arg Gly	
565 570 575	
GCC CAC GGC CAC AGC GTC CGC GGA AGC CCG AGG CAC AGG CCG AAG CGC	1776
Ala His Gly His Ser Val Arg Gly Ser Pro Arg His Arg Pro Lys Arg	
580 585 590	
TAC AGG CTC TTC ATA GAG TTC TTC GAG CGC AAG CTC AAG AAG TAC GAG	1824
Tyr Arg Leu Phe Ile Glu Phe Phe Glu Arg Lys Leu Lys Lys Tyr Glu	
595 600 605	
GAG GGC TTT GAG GTA GAG AAG ATA CTC AAG GGG AAT GGG AAC TGA	1869
Glu Gly Phe Glu Val Glu Lys Ile Leu Lys Gly Asn Gly Asn	
610 615 620	

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 622 AMINO ACIDS
 - (B) TYPE: AMINO ACID
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Thr	Gly	Ile	Glu	Trp	Asn	His	Glu	Thr	Phe	Ser	Lys	Phe	Ala	Tyr		
				5					10							15	
Leu	Gly	Asp	Pro	Arg	Ile	Arg	Gly	Asn	Leu	Ile	Ala	Tyr	Thr	Leu	Thr		
			20					25					30				
Lys	Ala	Asn	Met	Lys	Asp	Asn	Lys	Tyr	Glu	Ser	Thr	Val	Val	Val	Glu		
		35					40					45					
Asp	Leu	Glu	Thr	Gly	Ser	Arg	Arg	Phe	Ile	Glu	Asn	Ala	Ser	Met	Pro		
	50					55					60						
Arg	Ile	Ser	Pro	Asp	Gly	Arg	Lys	Leu	Ala	Phe	Thr	Cys	Phe	Asn	Glu		
65					70					75					80		
Glu	Lys	Lys	Glu	Thr	Glu	Ile	Trp	Val	Ala	Asp	Ile	Gln	Thr	Leu	Ser		
				85					90					95			
Ala	Lys	Lys	Val	Leu	Ser	Thr	Lys	Asn	Val	Arg	Ser	Met	Gln	Trp	Asn		
			100					105					110				
Asp	Asp	Ser	Arg	Arg	Leu	Leu	Val	Val	Gly	Phe	Lys	Arg	Arg	Asp	Asp		
		115				120						125					
Glu	Asp	Phe	Val	Phe	Asp	Asp	Asp	Val	Pro	Val	Trp	Phe	Asp	Asn	Met		
	130				135						140						
Gly	Phe	Phe	Asp	Gly	Glu	Lys	Thr	Thr	Phe	Trp	Val	Leu	Asp	Thr	Glu		
145					150					155					160		
Ala	Glu	Glu	Ile	Ile	Glu	Gln	Phe	Glu	Lys	Pro	Arg	Phe	Ser	Ser	Gly		
			165						170					175			
Leu	Trp	His	Gly	Asp	Ala	Ile	Val	Val	Asn	Val	Pro	His	Arg	Glu	Gly		
			180					185					190				
Ser	Lys	Pro	Ala	Leu	Phe	Lys	Phe	Tyr	Asp	Ile	Val	Leu	Trp	Lys	Asp		
		195					200					205					
Gly	Glu	Glu	Glu	Lys	Leu	Phe	Glu	Arg	Val	Ser	Phe	Glu	Ala	Val	Asp		
	210					215					220						
Ser	Asp	Gly	Lys	Arg	Ile	Leu	Leu	Arg	Gly	Lys	Lys	Lys	Lys	Arg	Phe		
225					230					235					240		
Ile	Ser	Glu	His	Asp	Trp	Leu	Tyr	Leu	Trp	Asp	Gly	Glu	Leu	Lys	Pro		
			245						250					255			
Ile	Tyr	Glu	Gly	Pro	Leu	Asp	Val	Trp	Glu	Ala	Lys	Leu	Thr	Glu	Gly		
		260						265					270				
Lys	Val	Tyr	Phe	Leu	Thr	Pro	Asp	Ala	Gly	Arg	Val	Asn	Leu	Trp	Leu		
		275					280					285					
Trp	Asp	Gly	Lys	Ala	Glu	Arg	Val	Val	Thr	Gly	Asp	His	Trp	Ile	Tyr		
	290					295					300						
Gly	Leu	Asp	Val	Ser	Asp	Gly	Lys	Ala	Leu	Leu	Leu	Ile	Met	Thr	Ala		
305	</																

- 43 -

Thr Arg Ile Gly Glu Leu Tyr Leu Tyr Asp Gly Glu Leu Lys Gln Val
 325 330 335
 Thr Glu Tyr Asn Gly Pro Ile Phe Arg Lys Leu Lys Thr Phe Glu Pro
 340 345 350
 Arg His Phe Arg Phe Lys Ser Lys Asp Leu Glu Ile Asp Gly Trp Tyr
 355 360 365
 Leu Arg Pro Glu Val Lys Glu Glu Lys Ala Pro Val Ile Val Phe Val
 370 375 380
 His Gly Gly Pro Lys Gly Met Tyr Gly His Arg Phe Val Tyr Glu Met
 385 390 395 400
 Gln Leu Met Ala Ser Lys Gly Tyr Tyr Val Val Phe Val Asn Pro Arg
 405 410 415
 Gly Ser Asp Gly Tyr Ser Glu Asp Phe Ala Leu Arg Val Leu Glu Arg
 420 425 430
 Thr Gly Leu Glu Asp Phe Glu Asp Ile Met Asn Gly Ile Glu Glu Phe
 435 440 445
 Phe Lys Leu Glu Pro Gln Ala Asp Arg Glu Arg Val Gly Ile Thr Gly
 450 455 460
 Ile Ser Tyr Gly Gly Phe Met Thr Asn Trp Ala Leu Thr Gln Ser Asp
 465 470 475 480
 Leu Phe Lys Ala Gly Ile Ser Glu Asn Gly Ile Ser Tyr Trp Leu Thr
 485 490 495
 Ser Tyr Ala Phe Ser Asp Ile Gly Leu Trp Tyr Asp Val Glu Val Ile
 500 505 510
 Gly Pro Asn Pro Leu Glu Asn Glu Asn Phe Arg Lys Leu Ser Pro Leu
 515 520 525
 Phe Tyr Ala Gln Asn Val Lys Ala Pro Ile Leu Leu Ile His Ser Leu
 530 535 540
 Glu Asp Tyr Arg Cys Pro Leu Asp Gln Ser Leu Met Phe Tyr Asn Val
 545 550 555 560
 Leu Lys Asp Met Gly Lys Glu Ala Tyr Ile Ala Ile Phe Lys Arg Gly
 565 570 575
 Ala His Gly His Ser Val Arg Gly Ser Pro Arg His Arg Pro Lys Arg
 580 585 590
 Tyr Arg Leu Phe Ile Glu Phe Phe Glu Arg Lys Leu Lys Lys Tyr Glu
 595 600 605
 Glu Gly Phe Glu Val Glu Lys Ile Leu Lys Gly Asn Gly Asn
 610 615 620

- 44 -

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 50 NUCLEOTIDES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR

- (ii) MOLECULE TYPE: Oligonucleotide

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCGAGAATTC ATTAAAGAGG AGAAATTAAC TATGACCGGC ATCGAATGGA

50

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 33 NUCLEOTIDES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR

- (ii) MOLECULE TYPE: Oligonucleotide

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATAAGGATC CAACTGGCA CAGTGTCAAG ACA

33

- 45 -

What Is Claimed Is:

1. An isolated polynucleotide which encodes the amino acid sequence set forth in SEQ ID NO:2.
2. An isolated polynucleotide selected from the group consisting of:
 - a) SEQ ID NO:1;
 - b) SEQ ID NO:1, wherein T can also be U;
 - c) nucleic acid sequences complementary to a) and b);
and
 - d) fragments of a), b), or c) that are at least 15 bases in length and that will hybridize to DNA which encodes the amino acid sequence of SEQ ID NO:2.
3. The polynucleotide of claim 1, wherein the polynucleotide is isolated from a prokaryote.
4. An expression vector including the polynucleotide of claim 1.
5. The vector of claim 4, wherein the vector is a plasmid.
6. The vector of claim 4, wherein the vector is a virus-derived.
7. A host cell transformed with the vector of claim 4.

- 46 -

8. The host cell of claim 7, wherein the cell is prokaryotic.
9. The polynucleotide of claim 1 which encodes the enzyme comprising amino acid 1 to 622 of SEQ ID NO:2.
10. The polynucleotide of claim 1 comprising the sequence as set forth in SEQ ID NO:1 from nucleotide 1 to nucleotide 1866.
11. A substantially pure polypeptide selected from the group consisting of:
 - a) an enzyme comprising an amino acid sequence which is at least 70% identical to the amino acid sequence set forth in SEQ ID NO:2;
 - b) an enzyme which comprises at least 30 amino acid residues to the enzyme of a); and
 - c) the amino acid sequence as set forth in SEQ ID NO:2.
12. Antibodies that bind to the polypeptide of claim 11.
13. The antibodies of claim 12, wherein the antibodies are polyclonal.
14. The antibodies of claim 12, wherein the antibodies are monoclonal.

- 47 -

15. A method for producing an enzyme comprising growing a host cell of claim 7 under conditions which allow the expression of the nucleic acid and isolating the enzyme encoded by the nucleic acid.
16. A process for producing a recombinant cell comprising transforming or transfecting the cell with the vector of claim 4 such that the cell expresses a polypeptide encoded by the DNA contained in the vector.
17. A process for removal of arginine phenylalanine or methionine from the N-terminal end of peptides in peptide or peptidomimetic synthesis, comprising: administering an amount of the enzyme of claim 10 effective for removal of arginine phenylalanine or methionine from the N-terminal end of peptides in peptide or peptidomimetic synthesis.

Figure 1

Thermococcus GU5L5 Amidase

1 ATG ACC GGC ATC GAA TGG AAC CAC GAG ACC TTT TCT AAG TTC GCC TAC
CTG GGC GAC CCG 60
1 Met Thr Gly Ile Glu Trp Asn His Glu Thr Phe Ser Lys Phe Ala Tyr
Leu Gly Asp Pro 20

61 AGG ATA CGG GGA AAC TTA ATC GCG TAC ACC CTG ACG AAG GCC AAC ATG
AAG GAC AAC AAG 120
21 Arg Ile Arg Gly Asn Leu Ile Ala Tyr Thr Leu Thr Lys Ala Asn Met
Lys Asp Asn Lys 40

121 TAC GAG AGC ACG GTT GTT GTT GAA GAC CTT GAA ACG GGC TCA AGG CGC
TTC ATC GAG AAC 180
41 Tyr Glu Ser Thr Val Val Val Glu Asp Leu Glu Thr Gly Ser Arg Arg
Phe Ile Glu Asn 60

181 GCC TCA ATG CCG AGG ATT TCG CCA GAC GGC AGA AAG CTC GCC TTC ACC
TGC TTT AAC GAG 240
61 Ala Ser Met Pro Arg Ile Ser Pro Asp Gly Arg Lys Leu Ala Phe Thr
Cys Phe Asn Glu 80

241 GAG AAG AAG GAG ACC GAG ATA TGG GTG GCC GAT ATC CAG ACC CTG AGC
GCC AAG AAA GTC 300
81 Glu Lys Lys Glu Thr Glu Ile Trp Val Ala Asp Ile Gln Thr Leu Ser
Ala Lys Lys Val 100

301 CTC TCA ACT AAA AAC GTC CGC TCG ATG CAG TGG AAC GAC GAT TCA AGG
AGA CTC TTA GTT 360
101 Leu Ser Thr Lys Asn Val Arg Ser Met Gln Trp Asn Asp Asp Ser Arg
Arg Leu Leu Val 120

361 GTC GGC TTC AAG AGG AGG GAC GAT GAG GAC TTC GTC TTT GAC GAC GAC
GTC CCG GTC TGG 420

121 Val Gly Phe Lys Arg Arg Asp Asp Glu Asp Phe Val Phe Asp Asp Asp
Val Pro Val Trp 140

421 TTC GAC AAT ATG GGA TTC TTT GAT GGA GAG AAG ACG ACG TTC TGG GTT
CTT GAC ACT GAG 480

141 Phe Asp Asn Met Gly Phe Phe Asp Gly Glu Lys Thr Thr Phe Trp Val
Leu Asp Thr Glu 160

481 GCC GAG GAG ATA ATC GAG CAG TTC GAG AAG CCG AGG TTT TCG AGT GGC
CTC TGG CAC GGC 540

161 Ala Glu Glu Ile Ile Glu Gln Phe Glu Lys Pro Arg Phe Ser Ser Gly
Leu Trp His Gly 180

541 GAT GCG ATA GTT GTG AAC GTC CCG CAC CGC GAG GGG AGC AAG CCT GCC
CTG TTC AAG TTC 600

181 Asp Ala Ile Val Val Asn Val Pro His Arg Glu Gly Ser Lys Pro Ala
Leu Phe Lys Phe 200

601 TAC GAC ATA GTC CTA TGG AAG GAC GGG GAG GAA GAG AAG CTC TTC GAG
AGG GTC TCC TTC 660

201 Tyr Asp Ile Val Leu Trp Lys Asp Gly Glu Glu Glu Lys Leu Phe Glu
Arg Val Ser Phe 220

661 GAG GCG GTT GAC TCC GAC GGA AAG AGA ATA CTC CTG AGG GGC AAG AAA
AAA AAG CGG TTC 720

221 Glu Ala Val Asp Ser Asp Gly Lys Arg Ile Leu Leu Arg Gly Lys Lys
Lys Lys Arg Phe 240

721 ATC AGC GAG CAC GAC TGG CTG TAC CTC TGG GAC GGC GAG CTT AAA CCG
ATC TAC GAG GGC 780

241 Ile Ser Glu His Asp Trp Leu Tyr Leu Trp Asp Gly Glu Leu Lys Pro
Ile Tyr Glu Gly 260

781 CCG CTC GAC GTC TGG GAA GCC AAG CTC ACG GAA GGA AAG GTC TAC TTC
CTC ACT CCA GAT 840

261 Pro Leu Asp Val Trp Glu Ala Lys Leu Thr Glu Gly Lys Val Tyr Phe
Leu Thr Pro Asp 280

841 GCG GGC AGG GTA AAC CTC TGG CTC TGG GAC GGG AAG GCC GAG CGT GTT
GTT ACC GGC GAC 900

281 Ala Gly Arg Val Asn Leu Trp Leu Trp Asp Gly Lys Ala Glu Arg Val
Val Thr Gly Asp 300

901 CAC TGG ATT TAC GGG CTT GAC GTC AGC GAT GGC AAA GCA TTG CTC CTC
ATC ATG ACC GCC 960

301 His Trp Ile Tyr Gly Leu Asp Val Ser Asp Gly Lys Ala Leu Leu Leu
Ile Met Thr Ala 320

961 ACG AGG ATA GGC GAG CTC TAC CTC TAC GAC GGC GAG CTG AAA CAG GTC
ACC GAA TAC AAC 1020

321 Thr Arg Ile Gly Glu Leu Tyr Leu Tyr Asp Gly Glu Leu Lys Gln Val
Thr Glu Tyr Asn 340

1021 GGG CCG ATA TTC AGG AAG CTC AAG ACC TTC GAG CCG AGG CAC TTC CGC
TTC AAG AGC AAA 1080

341 Gly Pro Ile Phe Arg Lys Leu Lys Thr Phe Glu Pro Arg His Phe Arg
Phe Lys Ser Lys 360

1081 GAC CTC GAG ATA GAC GGC TGG TAC CTC AGG CCG GAG GTT AAA GAG GAG
AAG GCC CCG GTG 1140

361 Asp Leu Glu Ile Asp Gly Trp Tyr Leu Arg Pro Glu Val Lys Glu Glu
Lys Ala Pro Val 380

1141 ATA GTC TTC GTC CAC GGC GGG CCG AAG GGC ATG TAC GGA CAC CGC TTC
GTC TAC GAG ATG 1200

381 Ile Val Phe Val His Gly Gly Pro Lys Gly Met Tyr Gly His Arg Phe
Val Tyr Glu Met 400

1201 CAG CTG ATG GCG AGC AAG GGC TAC TAC GTC GTC TTC GTG AAC CCG CGC
GGC AGC GAC GGC 1260

401 Gln Leu Met Ala Ser Lys Gly Tyr Tyr Val Val Phe Val Asn Pro Arg
Gly Ser Asp Gly 420

4/6

1261 TAT AGC GAA GAC TTC GCG CTC CGC GTC CTG GAG AGG ACT GGC TTG GAG
GAC TTT GAG GAC 1320

421 Tyr Ser Glu Asp Phe Ala Leu Arg Val Leu Glu Arg Thr Gly Leu Glu
Asp Phe Glu Asp 440

1321 ATA ATG AAC GGC ATC GAG GAG TTC TTC AAG CTC GAA CCG CAG GCC GAC
AGG GAG CGC GTT 1380

441 Ile Met Asn Gly Ile Glu Glu Phe Phe Lys Leu Glu Pro Gln Ala Asp
Arg Glu Arg Val 460

1381 GGA ATA ACG GGC ATA AGC TAC GGC GGC TTC ATG ACC AAC TGG GCC TTG
ACT CAG AGC GAC 1440

461 Gly Ile Thr Gly Ile Ser Tyr Gly Gly Phe Met Thr Asn Trp Ala Leu
Thr Gln Ser Asp 480

1441 CTC TTC AAG GCA GGA ATA AGC GAG AAC GGC ATA AGC TAC TGG CTC ACG
AGC TAC GCC TTC 1500

481 Leu Phe Lys Ala Gly Ile Ser Glu Asn Gly Ile Ser Tyr Trp Leu Thr
Ser Tyr Ala Phe 500

1501 TCG GAC ATA GGG CTC TGG TAC GAC GTC GAG GTC ATC GGG CCA AAT CCG
TTA GAG AAC GAG 1560

501 Ser Asp Ile Gly Leu Trp Tyr Asp Val Glu Val Ile Gly Pro Asn Pro
Leu Glu Asn Glu 520

1561 AAC TTC AGG AAG CTC AGC CCG CTG TTC TAC GCT CAG AAC GTG AAG GCG
CCG ATA CTC CTA 1620

521 Asn Phe Arg Lys Leu Ser Pro Leu Phe Tyr Ala Gln Asn Val Lys Ala
Pro Ile Leu Leu 540

1621 ATC CAC TCG CTT GAG GAC TAC CGC TGT CCG CTC GAC CAG AGC CTT ATG
TTC TAC AAC GTG 1680

541 Ile His Ser Leu Glu Asp Tyr Arg Cys Pro Leu Asp Gln Ser Leu Met
Phe Tyr Asn Val 560

1681 CTC AAG GAC ATG GGC AAG GAA GCC TAC ATA GCG ATA TTC AAG CGC GGC
GCC CAC GGC CAC 1740

5/6

561 Leu Lys Asp Met Gly Lys Glu Ala Tyr Ile Ala Ile Phe Lys Arg Gly
Ala His Gly His 580

1741 AGC GTC CGC GGA AGC CCG TGG CAC AGG CCG AAG CGC TAC AGG CTC TTC
ATA GAG TTC TTC 1800

581 Ser Val Arg Gly Ser Pro Arg His Arg Pro Lys Arg Tyr Arg Leu Phe
Ile Glu Phe Phe 600

1801 GAG CGC AAG CTC AAG AAG TAC GAG GAG GGC TTT GAG GTA GAG AAG ATA
CTC AAG GGG AAT 1860

601 Glu Arg Lys Leu Lys Lys Tyr Glu Glu Gly Phe Glu Val Glu Lys Ile
Leu Lys Gly Asn 620

1861 GGG AAC TGA 1869

621 Gly Asn End 623

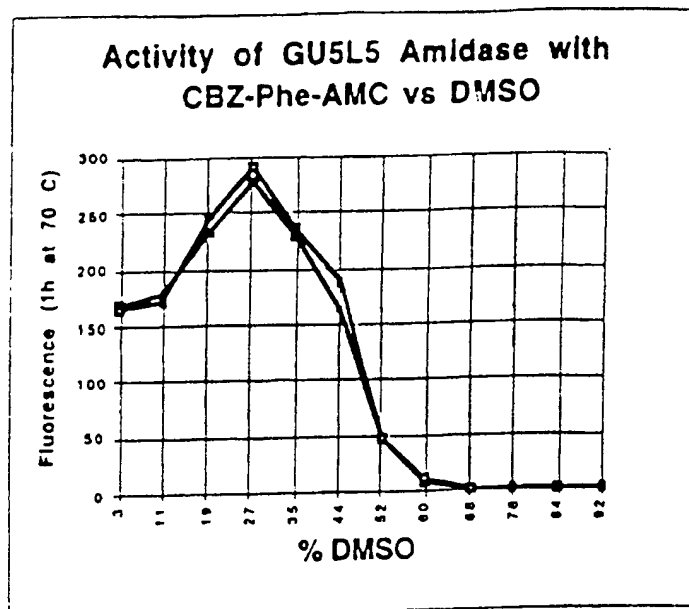


Figure 2

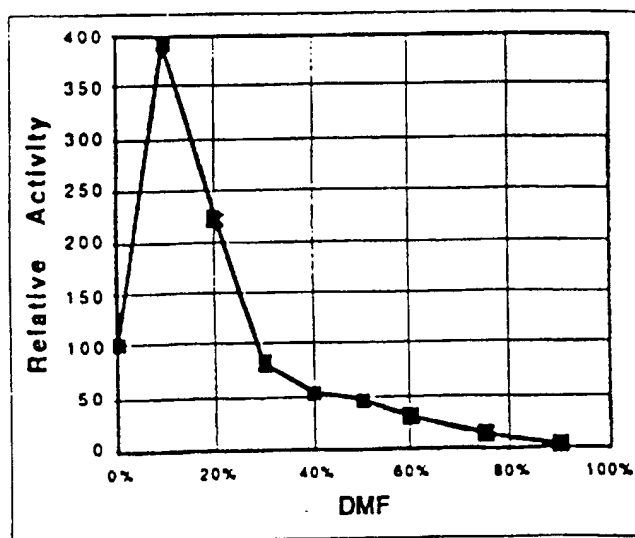


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09319

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/80, 15/00, 1/20; C12P 21/06; C07H 21/04; C07K 16/00

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/228, 69.1, 252.3, 320.1, 68.1; 536/23.2, 23.7; 530/387.1, 388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

MPurch pp - protein database search - geneseq25.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^a	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,451,522 A (QUEENER et al.) 19 September 1995, see entire document.	1-17



Further documents are listed in the continuation of Box C.



See patent family annex.

^a

Special categories of cited documents:

A

document defining the general state of the art which is not considered to be of particular relevance

E

earlier document published on or after the international filing date

L

document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O

document referring to an oral disclosure, use, exhibition or other means

P

document published prior to the international filing date but later than the priority date claimed

T

later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention seems to be considered novel or seems to be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention seems to be considered to involve an inventive step when the document is considered with one or more other such documents, such combination being obvious to a person skilled in the art

Z

documents members of the same patent family

Date of the actual completion of the international search

11 AUGUST 1997

Date of mailing of the international search report

03 SEP 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Tetchand Saidha

Telephone No. (703) 308-0196